Section II (Remarks)

Amendment of Claims to More Specifically Recite the Added Character of the Small Heat Shock Protein

Claims 1, 3, 4 and 6 have been amended herein to recite that the small heat shock protein is small heat shock protein (sHSP) "**added to said protein susceptible to degradation by protease**" in the recited amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample composition," and claim 7 has correspondingly been amended to recite "added" in reference to the recited small heat shock protein.

These amendments make it clear that the small heat shock protein is "**added**" to the "**protein susceptible to degradation by protease,**" consistent with the supporting description in the application at page 6, lines 1-2 ("adding sHSPs to the protein mixture") and page 6, lines 23-26 ("the amount of the sHSPs that is added ... is preferably in a range of 0.1 to 50 parts by weight, and more preferably 0.5 to 20 parts by weight, relative to 100 parts by weight of the total protein of an electrophoresis sample").

Resolution of 35 USC §112 Issues

Compliance of Claims as Amended with Requirements of 35 USC §112, Second Paragraph

In the April 19, 2006 Office Action., claims 1, 3, 4, 6-11 and 18 were rejected[1] under 35 USC §112 for indefiniteness.

The examiner in the April 19, 2006 Office Action in reference to these claims has stated that

"the possible maximum amount of proteins that could be used at the same time in the method is not provided or particularly pointed out (whether forty one or only one protein is used at the

[1] Although the statement of rejection of page 2 of the Office Action applied the 35 USC §112, second paragraph rejection to claims "1, 3, 4, 6-11, and 18," it is to be noted that claim 8 was cancelled in the Response filed on February 2, 2006 to the November 2, 2005 Office Action in this application. Claim 8 therefore should not have been identified in the statement of rejected claims.

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same time in the method claimed). Therefore, the clarification as to the number of proteins should be provided."

This statement refers to the recital that is for example set out in claim 1:

"small heat shock protein (sHSP) in an amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample composition, wherein said sHSP includes at least one of the forty HSPs selected from the group consisting of:
IbpA (inclusion body-associated protein A) derived from *Agrobacterium tumefaciens*;
sHSPs derived from *Arabidopsis thaliana*;
HspB (heat shock protein B), HspH (heat shock protein H), HspC (heat shock protein C) and HspF (heat shock protein F) derived from *Bradyrbizobium japonicum*; ...."

This claim language is submitted to be fully clear on its face, since it is apparent that 40 heat shock protein species are recited, and the language "at least one" makes clear that the composition of applicants' claimed invention may contain one, two, three, etc. up to all forty of such heat shock protein species, in specific embodiments of the claimed invention.

It is useful in this respect to review the history of this application. The originally filed claim 2 of the application recited that the heat shock proteins in the claimed composition "are one or more selected from proteins set forth in Table 1" as the "effective amount" of small heat shock proteins recited in originally filed claim 1.

In the November 2, 2005 Office Action, the examiner rejected claim 1 as reciting "at least one" small heat shock protein and stated that "the maximum possible amount of proteins is not provided or particularly pointed out...[T]herefore, the clarification as to the number of proteins should be provided."

In the response filed February 2, 2006 to such November 2, 2005 Office Action, claim 1 was amended to recite "at least one of the forty HSPs selected from the group consisting of..."[followed by the recital of the individual heat shock protein species]."

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The examiner in the present Office Action has maintained that the "possible maximum .. is not provided or particularly pointed out (whether forty one or only one protein is used at the same time in the method claimed)."

The indefiniteness alleged by the examiner to exist in this claim language is not perceived to be present by applicants. Taking a simplified case, if a claim recites in a composition the presence of at least one of species A, species B and species C, then it is apparent that the composition may in various embodiments within the scope of such claim:

- contain only one of the three identified species – thus, the claim covers a composition in which only species A is present; alternatively, it covers a composition in which only species B is present; and as a still further alternative, it covers a composition in which only species C is present;
- contain two of the three identified species – thus, the claim covers a composition in which A and B are present together, or a composition in which A and C are present together, or a composition in which B and C are present together, as specific embodiments of the claimed composition; or
- contain all three of the three identified species – thus, the claim covers a composition in which species, A, B and C are all present in the composition.

This, then, is the plain meaning of the terminology at issue. One, two, three, etc., up to a maximum of ALL of the recited species is contemplated and encompassed by the claim language. Applicant has attempted fully to comply with the examiner's requirement for definiteness by being quantitatively precise.

Therefore, in the face of the examiner's continued rejection of the claims as allegedly lacking a "maximum ...number of proteins," despite the fact that ALL of the recited heat shock protein species can be employed together, and despite the fact that ALL of the recited heat shock protein species together constitute a maximum number, claim 1 has been amended to recite the sHSP in simple and classical Markush language:

> **"wherein said added sHSP includes sHSP selected from the group consisting of: ...",**

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thereby establishing the subsequently recited sHSP species as a selection group from which small heat shock protein can be chosen, to select ONE or MORE, up to ALL, of the recited sHSP species as the sHSP of the claimed composition, within the scope of applicants' claimed invention.

Claims 3, 4, 6, 7, 9, 10 and 11 have been correspondingly amended.

The language "wherein said sHSP includes sHSP selected from the group consisting of: ..." is clear and definite on its face, and comports with the requirements of MPEP 803.02 (i.e., being of the form "selected from the group consisting of A, B and C." See *Ex parte Markush*, 1925 C.D. 126 (Comm'r Pat. 1925)).

It therefore is submitted that the claims are fully clear and definite within the requirements of 35 USC §112, second paragraph.

Compliance of Claims as Amended with Requirements of 35 USC §112, First Paragraph

Claims 7 and 18 were rejected in the April 19, 2006 Office Action, as allegedly failing to comply with the written description requirement of 35 USC §112, first paragraph based on the recital of "at least 50% increased number of spots."

Although such at least 50% increased number of spots is shown in various electrophoretic gels in the drawings of the application, e.g., FIG. 11 gels (C) (added IbpA) and (D) (added HSP26), in relation to gel (A) (control), and although drawings have status as disclosure – see MPEP 608 ("Disclosure"), stating that **"Applicant may rely for disclosure upon the specification with original claims and drawings, as filed"** – to resolve the examiner's objection and advance the application to allowance, applicants have amended claims 7, 9 and 18 to delete the recital of "at least 50%" from such claims, and to specify in claim 7 that the small heat shock protein is "added" small heat shock protein. The amended claim 7 now recites, *inter alia*,

> **"adding small heat shock protein (sHSP) to the sample composition in an amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample**

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> **composition, so as to prevent protein degradation and obtain a gel with an increased number of spots as compared to a gel obtained for a corresponding sample composition lacking said added small heat shock protein**",

thereby making fully clear that the number of spots – a readily visually ascertainable feature of the electrophoretic gels – in compositions containing added small heat shock protein, is quantitatively greater than the number of spots on electrophoretic gels of corresponding compositions lacking the added small heat shock protein. See, for example, the FIG. 11 gels referred to above, wherein the spot population in the samples (C) and (D) treated with added heat shock protein is much greater than in the control sample (A) to which no added small heat shock protein was introduced.

See also the text of the application, e.g., at page 20, lines 12-14 (describing software commercially available from Bio-Rad Laboratories for measuring the number of protein spots on electrophoretic gels, thereby reflecting that the methodology of claims 7 and 16 is enabled by commercially available tools permitting one to quantitatively ascertain the improvement (increase of protein spots) afforded by adding small heat shock protein to a gel electrophoresis sample, in accordance with the applicants' invention.

See also Examples 4-7 of the specification, and the accompanying FIGS. 8 and 10-13, respectively discussing and pictorially showing the increase in protein spots attributable to the adding of small heat shock protein to the electrophoretic gel samples, as compared to corresponding samples lacking such added small heat shock protein.

In connection with the foregoing, attached in Appendix A hereof is a publication whose co-authors include inventors Sung Yup Lee and Mee-Jung Han, entitled "Enhanced Proteome Profiling by Inhibiting Proteolysis with Small Heat Shock Proteins," Journal of Proteome Research 2005, 4, 2429-2434, American Chemical Society, published on the Web on October 21, 2005. The Examiner's attention in such publication is directed to page 2433, in the left panel at line 15 to the right panel at line 4, in relation to FIGS. 10 and 11 of the present application, in which FIG. 10 represented entire 2D gels and FIG. 11 represents a part of the 2D gels of FIG. 10.

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Based on the publication, in FIG. 10, (A) represents 900 spots, (E) represents 1,200 spots, (F) represents 1,600 spots, and (I) represents 1,700 spots. As compared with control FIG. 10 (A) – without treatment with protease inhibitor the group with added IbpA (FIG. 10 (F)) and the group with added HSP26 (FIG. 10 (I)) have spots increased by 78% and 89%, respectively. In addition, as compared with the group with an added cocktail inhibitor (FIG. 10 (E)), the group with added IbpA (FIG. 10 (F)) and the group with added HSP26 (FIG. 10 (I)) have spots increased by 33% and 42%, respectively.

Claims 7, 9 and 18 as amended fully comply with 35 USC §112, first paragraph.

Rejections of Claims Based on Lubman, Willsie and Kitagawa, and Traversal Thereof

Claims 1, 3, 4, 6, 7, 9-11, 13-15 and 19 were rejected in the April 19, 2006 Office Action, as allegedly obvious over the prior art.

Specifically,

- claims 1 and 15 were rejected under 35 USC §103 as obvious over Lubman (Lubman, et al., U.S. No. 2002/0098595 A1), and
- claims 1, 3, 4, 6, 7, 10, 11, 13 and 14 were rejected as obvious over Willsie (Willsie et al., *J. of Cellular Biochem.*, (2002), 84, p. 601-614) in view of Kitagawa (Kitagawa, et al., Eur. J. Biochem. (2002), 269, p. 2907-2917).

These rejections are traversed. The following discussion clearly shows all pending claims to be patentable over the references that have been cited against them.

Patentability of Claims 1 and 15 Over Lubman

Lubman has been cited in the April 19, 2006 Office Action to:

- "teach that heat shock proteins have been identified from the 2-D gel" (the examiner citing paragraph [0137] at page 15 of Lubman) and

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- "provide a method analogous to the 2-D gel, where the method can be used for proteome analysis" (the examiner citing paragraph [0151] at page 17 of Lubman).

From such disclosure, the examiner has stated (page 4 of the April 19, 2006 Office Action) that

> **"it would have been obvious to a person skilled in the art to design a method for the analysis of proteomes using 2-D gel electrophoresis where the method is analogous to the 2-D gel and uses heat shock proteins as disclosed by Lubman et al."**

This conclusion is respectfully traversed.

As disclosed at page 3, lines 7-10 of the present application,

> **"heat shock proteins (HSPs) with a low molecular weight of 15-30 kDa are induced by stress such as heat shock or the overproduction of certain proteins, and .... are present in each of all organisms from eukaryotes to prokaryotes"**

Thus, in the process methodology described in Lubman involving "multi-phase protein separation methods capable of resolving and characterizing large numbers of cellular proteins" (Lubman, Abstract, lines 1-3; emphasis added), the cellular protein samples may contain heat shock proteins that are incidentally present as endogenous components of the cellular protein sample being assayed, but there is no teaching, suggestion or derivative basis in Lubman of any methodology in which "added" small heat shock protein (i.e., heat shock protein that is exogenous to the cellular sample being assayed) is introduced to the cellular protein sample, in contrast to applicants' claimed invention, which requires, *inter alia*, a

> **"sample composition containing small heat shock protein (sHSP) added to said protein susceptible to degradation by protease, in an amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample composition"** (claim 1; emphasis added).

Lubman therefore fails to provide any disclosure of adding small heat shock protein to a sample, as opposed to resolving or detecting small heat shock protein that may already be intrinsically present in a cellular protein sample.

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Moreover, the amounts of small heat shock proteins that would be intrinsically present in a cellular sample as endogenous sHSP would be << 0.1 wt. %, further underscoring the lack of any relevant basis in Lubman for the applicants' invention as claimed, since applicants' claimed invention requires at least 0.1 wt. % small heat shock protein to be "**added to said protein susceptible to degradation by protease**" (instant claim 1).

Since Lubman does not contemplate adding small heat shock protein to a protein sample, Lubman therefore cannot contemplate the addition of such additive in an amount of 0.1-50% that is required by applicants' claims.

Three requirements must be met to support a *prima facie* case of obviousness, as set out in MPEP § 2143:

> **2143 Basic Requirements of a *Prima Facie* Case of Obviousness**
>
> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there **must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference** or to combine reference teachings. Second, there must be a **reasonable expectation of success**. Finally, the **prior art reference (or references when combined) must teach or suggest all the claim limitations**.
>
> The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).
>
> MPEP § 2143 (emphasis added)

Since Lubman (i) contains no suggestion or motivation to introduce added heat shock protein to a protein sample susceptible to degradation by protease, (ii) fails to evidence any awareness of the electrophoretic gel "spots issue" faced and overcome by the applicants, and (iii) contains no disclosure of adding 0.1-50 wt.% sHSP to a protein sample susceptible to degradation by protease, it is apparent that Lubman fails to satisfy the requirements for §103 rejection, and that

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applicants' claim 1, as well as applicants' claim 15 (the latter requiring "using the composition of claim 1") are fully patentably distinguished over Lubman.

Patentability of Claims 1, 3, 4, 6, 7, 10, 11 and 13-14 Over Willsie in View of Kitagawa

In the rejection of claims 1, 3, 4, 6, 7, 10, 11 and 13-14 over Willsie in view of Kitigawa, Willsie has been cited as teaching α-crystalin protein p26 HSP bound to nuclear matrix proteins derived from embryos that were subject to 2-D gel electrophoresis.

The statement in the April 19, 2006 Office Action that "Willsie et al. teach a method of 2-D gel electrophoresis comprising adding small HSPs to a nuclear matrix protein mixture and subjecting the mixture to 2-D gel electrophoresis" misdescribes the empirical procedure of Willsie.

Willsie is in fact a mechanism study of intracellular movement of heat shock protein in brine shrimp embryos, involving translation of heat shock protein p26 from the cytoplasm into the nuclear matrix of the encysted embryos. See, for example, page 603, left column, lines 4-6 of Willsie:

> **"Three treatments were used to transfer p26 into nuclei of activated embryos: heat shock, anoxia in vivo, and acidic pH in vitro."**

In this work, the embryos were processed to effect transfer of the p26 heat shock protein into the nucleus of embryo cells, prior to homogenizing the embryos. For example, the heat shock used a temperature of 50°C, and Willsie discloses at page 603, left column, lines 12-14 that

> **"The temperature of 50°C was selected because this translocates the maximum amount of p26 into nuclei without killing any embryos"**

Once the nuclear translocation (movement of the heat shock protein from the cytoplasm to the nucleus) has taken place, Willsie fractionates the nuclei of the embryo cells and isolates the nuclear matrix, in order to determine the associations formed in the nucleus by the relocated p26 heat shock protein – this is the objective of the Willsie experimentation, resulting in the finding

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reflected in the title of the Willsie article (viz., "Small Heat Shock Protein p26 Associates With Nuclear Lamins and HSP70 in Nuclei and Nuclear Matrix Fractions From Stressed Cells").

Contrary to the statement in the April 19, 2006 Office Action, there is NO teaching in Willsie of "adding small HSPs to a nuclear matrix protein mixture and subjecting the mixture to 2-D gel electrophoresis." Instead, Willsie treats shrimp embryos to cause movement of p26 HSP from the cytoplasm into the nucleus of the embryo cells, then Willsie fractionates the nucleus of such cells, and assays the protein mixture of the fractionated nuclear material. WILLSIE DOES NOT ADD ANY SMALL HEAT SHOCK PROTEINS TO THE PROTEIN MIXTURE OF THE FRACTIONATED NUCLEAR MATERIAL. There is no "addition" at all – Willsie is processing whole embryos to "fix" endogenous heat shock proteins in the nucleus, and the only protein mixture that is processed by Willsie is one to which no additional heat shock protein is introduced. Willsie therefore teaches away from applicants' claimed invention, which requires, *inter alia*, a

> **"sample composition containing small heat shock protein (sHSP) added to said protein susceptible to degradation by protease, in an amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample composition"** (claim 1; emphasis added).

and Willsie correspondingly teaches away from the corresponding methodology of applicants' claimed invention requiring the provision of a gel electrophoresis sample composition comprising protein that is susceptible to degradation by protease, with protease present in the sample composition, and

> **"adding small heat shock protein (sHSP) to the sample composition in an amount in a range of from 0.1 to 50 parts, relative to 100 parts by weight of total protein in said sample composition,"**

as recited in applicants' claim 7.

Since Willsie fails to teach, suggest or in any way establish a basis for providing a composition or methodology of applicants' claimed invention, the disclosure of Kitigawa relating to specific heat shock proteins of E. coli, IbpA and IbpB, has no relevance to Willsie (except through a hindsight-motivated effort to patch together the applicants' claimed invention based solely on the

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applicants' own disclosure), since Willsie is a characterization study of translocated endogenous heat shock protein, p26, that is different from the IbpA and IbpB heat shock protein species of Kitigawa.

Since Willsie is specifically directed to study of the native heat shock protein p26 of encysted embryos of brine shrimp Artemia franciscana, where is the basis for the importation of the E.coli heat shock proteins IbpA and IbpB into such shrimp embryos, as urged to be *prima facie* obvious and constituting the basis that is hypothesized in the April 19, 2006 Office Action for the rejection of applicants' claims 1, 3, 4, 6, 7, 10, 11 and 13-14? The reason given in the Office Action is "because small heat shock proteins help to stabilize other proteins and remain associated with unfolded proteins in 2-D gel electrophoresis" (April 19, 2006 Office Action, page 6, lines 1-3).

The Office Action by this hypothesis appears to be suggesting that the E.Coli IbpA and IbpB HSPs of Kitigawa be substituted for the brine shrimp p26 HSP of Willsie, e.g., by a recombinant manipulation of the Willsie brine shrimp, but such substitution, in addition to being nowhere disclosed or suggested by the Willsie or Kitigawa references themselves, would logically have a high probability of a poorer effect on the stability and viability of the brine shrimp organism than the "native" p26 HSP that has evolved through natural selection and demonstrated superiority in enabling the *Artemia franciscana* embryo to accommodate temperatures as high as 50°C without dying (see disclosure in Willsie at page 603, left column, line 14 – "without killing any embryos").

The foregoing therefore shows that there is in fact no "reasonable expectation of success" (see again MPEP 2143) in the hypothetical combination of Willsie and Kitigawa – quite the contrary, there is the expectation that substituting the IbpA/IbpB HSPs for Willsie's p26 would result in a poorer result.

As a consequence, the combination of Willsie and Kitigawa fails to support a *prima facie* case of obviousness. The rejection of claims 1, 3, 4, 6, 7, 10, 11 and 13-14 therefore merits withdrawal, and the allowance of such claims is urged, inasmuch as there is no derivative basis in the cited prior art references for the composition and method of the applicants' pending claims.

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CONCLUSION

Based on the foregoing, all of applicants' now-pending amended claims 1, 3, 4, 6, 7, 9-11, 13-15 and 18 are patentably distinguished over the art, and in form and condition for allowance. Issue of a Notice of Allowance for the application is requested.

If any issues remain outstanding, incident to the allowance of the application, the examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss their resolution, in order that this application may be passed to issue at an early date.

Respectfully submitted,

Steven J. Hultquist
Reg. No. 28,021
Attorney for Applicants

INTELLECTUAL PROPERTY/
TECHNOLOGY LAW
Phone: (919) 419-9350
Fax: (919) 419-9354
Attorney File No.: 4240-103

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APPENDIX A

technical notes Journal of proteome research

Enhanced Proteome Profiling by Inhibiting Proteolysis with Small Heat Shock Proteins

Mee-Jung Han,[†] Jeong Wook Lee,[†] and Sang Yup Lee*[,†,‡]

Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical & Biomolecular Engineering and BioProcess Engineering Research Center, Daejeon, Republic of Korea, and Department of BioSystems and Bioinformatics Research Center, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

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Abstract: Proteolytic degradation is one of the critical problems in two-dimensional electrophoresis (2-DE). Here we report that small heat shock proteins (sHsps), including $IbpA_{Ec}$ and $IbpB_{Ec}$ from *Escherichia coli* and $Hsp26_{Sc}$ from *Saccharomyces cerevisiae*, are able to protect proteins in vitro from proteolytic degradation. Addition of sHsps during 2-DE of human serum or whole cell extracts of *E. coli*, *Mannheimia succiniproducens*, *Arabidopsis thaliana*, and human kidney cells allowed detection of up to 50% more protein spots than those obtainable with currently available protease inhibitors. Therefore, the use of sHsps during 2-DE significantly improves proteome profiling by generally enabling the detection of many more protein spots that could not be seen previously.

Keywords: small heat shock proteins • two-dimensional gel electrophoresis • proteome profiling • protease inhibitor

Introduction

Proteolytic degradation of proteins is one of the most important problems in two-dimensional electrophoresis (2-DE) of proteomic samples because it reduces the number of proteins that can be reproducibly observed. Proteolysis can also cause the appearance of protein spot artifacts, which can lead to incorrect conclusions about cellular biochemical changes, such as protein turnover, post-translational processing, and alternative gene splicing. Several strategies have been devised to solve these problems, including the addition of protease inhibitors, Tris base, or large amounts of dithiothreitol (DTT) and thiourea or urea; precipitation of proteins by trichloroacetic acid in acetone; and boiling the sample in sodium dodecyl sulfate (SDS)-containing sample buffer.[1−3] However, certain proteases still remain active even under these harsh conditions. Because there is no general strategy for preventing proteolysis for all kinds of samples, a variety of sample buffers and procedures must be tested. Loss of protein spots in 2-D gels due to residual protease activity is commonly observed when using immobilized pH gradient gels (IPGs) for isoelectric focusing (IEF).[1,2] In this case, proteolysis has commonly been reduced by adding protease inhibitors (PIs) to the reswelling buffer and reducing the time of protein incubation in this buffer. However, despite these steps, problems from proteolysis persisted.

The small heat shock proteins (sHsps) are molecular chaperones of 15−30 kDa found in prokaryotes and eukaryotes.[4,5] It has been shown that sHsps prevent irreversible aggregation of denatured proteins, and facilitate their transfer to other chaperone systems for correct refolding.[4−7] We have recently demonstrated that *E. coli* sHsps can protect recombinant proteins from degradation by cytoplasmic proteases in vivo.[7] In the current study, we examined whether sHsps can protect proteins from proteolysis in vitro. Three sHsps, specifically $IbpA_{Ec}$ and $IbpB_{Ec}$ from *E. coli* and $Hsp26_{Sc}$ from *S. cerevisiae*, were evaluated for their ability to do so during 2-DE.

Experimental Section

Gene Manipulation. *E. coli* XL1-Blue (*supE44 hsdR17 recA1 gyrA96 thi relA1 lac F′* [*proAB*$^{+}$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^{r})]; Stratagene Cloning Systems, La Jolla, CA) was used as a host strain for the cloning and maintenance of plasmids and for gene expression. Plasmids pTac99IbpA*his*$_{Ec}$, pTac99IbpB*his*$_{Ec}$, and pTac99Hsp26*his*$_{Sc}$ were employed for the production of sHsps. They were constructed by cloning the PCR-amplified, C-terminally His_6-tagged *E. coli ibpA*$_{Ec}$, *E. coli ibpB*$_{Ec}$, and *S. cerevisiae hsp26*$_{Sc}$ genes, respectively, into the *Eco*RI and *Hin*dIII sites of pTac99A.[8] Further details are provided in the Supporting Information online.

PCR was performed in a PCR Thermal Cycler MP (Takara Shuzo Ltd., Shiga, Japan) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing was carried out using the BigDye Terminator cycle sequencing kit (Perkin-Elmer, Boston, MA), Taq polymerase, and ABI Prism 377 DNA sequencer (Perkin-Elmer). All DNA manipulations were carried out according to standard procedures.[9]

Production and Purification of sHsps. Recombinant *E. coli* strains were cultivated in 250-mL flasks containing 100 mL Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with 50 mg/L ampicillin in a shaking

* To whom correspondence should be addressed. Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea. Phone: +82-42-869-3930. Fax: +82-42-869-3910. E-mail: leesy@kaist.ac.kr.

† Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical & Biomolecular Engineering and BioProcess Engineering Research Center.

‡ Department of BioSystems and Bioinformatics Research Center, Korea Advanced Institute of Science and Technology.

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incubator at 37 °C and 200 rpm. Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) with a DU Series 600 spectrophotometer (Beckman, Fullerton, CA). When the OD_{600} reached 0.7, cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO). After further cultivation for 4 h, cells were harvested by centrifugation at 3500 × *g* for 5 min at 4 °C. Recombinant sHsps were purified using a Ni-nitrilotriacetic acid spin column kit (Qiagen, Valencia, CA) under denaturing conditions. The amounts of purified sHsps were determined by Bradford assay using bovine serum albumin as a standard.[10] Purification of sHsps was confirmed by SDS polyacrylamide gel electrophoresis (PAGE) on a 15% (w/v) polyacrylamide gel as described by Laemmli.[11] Gels were stained with Coomassie brilliant blue R250 (Bio-Rad, Hercules, CA), and the protein bands were quantified by a GS-710 Calibrated Imaging Densitometer (Bio-Rad). To renature denatured proteins, protein fractions were dialyzed in several steps against 20 mM Tris-HCl buffer (pH 8.0) containing decreasing concentrations of urea (6 to 0 M). Refolding was confirmed by circular dichroism (see Supporting Information S2 online).

Evaluation of Proteolysis Inhibition. Human serum albumin (HSA; 66.5 kDa) was used as a model substrate protein at a concentration of 0.5 μg/μL. At time zero, trypsin (Boehringer Mannheim, Mannheim, Germany) or proteinase K (Sigma) was added at varying concentrations, and the reaction mixture was kept at room temperature for 2 h. After the reaction, aliquots (10 μL) were mixed with 10 μL of Laemmli sample buffer, followed by immediate SDS-PAGE on a 15% (w/v) polyacrylamide gel. A reference sample incubated with the same volume of buffer without protease was included as a control in all experiments. To compare the inhibition of proteolysis, PIs and sHsps were added to the HSA solution just before the addition of proteases and at the following concentrations: 1 mM PMSF (Sigma), 4 mM AEBSF (Pefabloc SC; Roche Diagnostics GmbH, Germany), 1 mM EDTA (Sigma), 0.1 tablet/mL of protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics GmbH, Germany), 0.005 μg/μL $IbpA_{Ec}$, 0.005 μg/μL $IbpB_{Ec}$, and 0.005 μg/μL $Hsp26_{Sc}$. These concentrations of commercial PIs were those recommended by 2-D brochure of Amersham Biosciences (Uppsala, Sweden).

Protein Extraction and Analysis by 2-DE. Proteome analysis was performed by 2-DE using the IPGphor IEF system (Amersham Biosciences) and Protean II xi Cell (Bio-Rad) as described previously.[7,12] In brief, *E. coli* W3110 or *M. succiniciproducens* MBEL55E (Korean Collection for Type Cultures, Daejeon, Korea) cells were harvested at the mid-logarithmic phase by centrifugation for 5 min at 3500 × *g* and 4 °C and washed four times with low-salt washing buffer. The pellet was then resuspended in 600 μL of a buffer containing 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.1% SDS. One μL of this sample was mixed with 60 μL of a solution consisting of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 65 mM DTT, and a trace of bromophenol blue. Proteins (200 μg for *E. coli* or 150 μg for *M. succiniciproducens*) quantified by Bradford assay[10] were resuspended in 350 μL of IEF denaturation buffer (8 M urea, 2% (w/v) CHAPS, 20 mM DTT, and 0.8% (v/v) IPG buffer). The sHsps as well as protease inhibitors were added at the concentrations indicated, and the samples were carefully loaded on the IPG strips (18 cm, pH 3−10 NL; Amersham Biosciences). The loaded IPG strips were rehydrated for 12 h and were focused at 20 °C for 15 min at 250 V, followed by 8000 V until a total of 60 000 V·h was reached. The strips were equilibrated in equilibration buffers and then were placed on 12% (w/v) SDS−PAGE gels prepared by the standard protocol.[11] Protein spots were visualized using a silver staining kit (Amersham Biosciences), and the stained gels were scanned by a GS-710 Calibrated Imaging Densitometer (Bio-Rad). The spots in a gel are automatically detected by ImageMaster 2D Platinum Software (version 5.0; Amersham Biosciences) with the optimal parameters of Smooth (2), Min Area (5) and Saliency (200).

Human serum was obtained from the Cancer Metastasis Research Center (Seoul, Republic of Korea) and was treated according to the protocol by Quero et al.[13] with some modifications. A serum sample (20 μL) diluted to 600 μL with 0.1 M PBS (pH 7.0) was sonicated on ice with a VibraCell (Sonics & Materials, Newtown, CT). Samples were precipitated by adding an equal volume of 20% (w/v) trichloroacetic acid in acetone at 4 °C for 30 min. After centrifugation at 16 100 × *g* for 30 min at 4 °C, the pellet was rinsed twice with ice-cold acetone. The pellet was air-dried and fully dissolved in 350 μL of IEF denaturation buffer. All other steps for 2-DE were as described above except that 150 μg of sample was analyzed, and IEF was carried out with an initial focusing at 100 V for 3 h, followed by a gradual increase to 1000 V over 6 h, and, finally, 8000 V for 2 h (60 000 total V·h).

The human kidney cell line 293 c18 (American Type Culture Collection, Manassas, VA) was rinsed once with Dulbecco's Modified Eagle Medium (GIBCO−Invitrogen, Carlsbad, CA) and removed from the flask with a mixture of 0.5 g/L trypsin and 0.2 g/L EDTA. After 3 min, medium containing fetal calf serum was added to the flask to terminate trypsinization. Cells were collected by centrifugation for 5 min at 3000 × *g*, resuspended in ice-cold PBS (pH 7.2), and collected again by centrifugation for 15 min at 3000 × *g*. Cells (8×10^5) were solubilized with 60 μL of a solution containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, and 65 mM DTT. 2-DE was carried out as described for the *E. coli* sample except that 150 μg of protein sample was analyzed.

A. thaliana Colombia[14] was treated as described by Carpentier et al.[15] with some modifications. Freshly prepared leaves (300 mg) were ground in a mortar with liquid nitrogen and mixed with 4 mL of 20% (w/v) trichloroacetic acid and 0.2% (w/v) DTT in acetone at −20 °C. The precipitated protein was collected by centrifugation at 16 100 × *g* for 30 min at 4 °C, and the pellet was washed with ice-cold 0.2% (w/v) DTT in acetone, incubated for 1 h at −20 °C, and washed again with ice-cold 0.2% (w/v) DTT in acetone. The resulting pellet was air-dried and dissolved in 200 μL of extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, and 0.8% (v/v) IPG buffer). Proteins were extracted for 1 h at 25 °C with vigorous vortexing. The extracted sample was diluted with rehydration buffer (6 M urea, 2 M thiourea, 0.5% (w/v) CHAPS, 30% (w/v) glycerol, 0.5% (v/v) IPG buffer, and 0.28% (w/v) DTT). 2-DE was carried out as described above except that 150 μg of protein sample was analyzed and IEF was performed with an initial focusing for 3 h at 300 V, followed by a gradual increase to 1000 V over 6 h, and, finally, 8000 V for 3 h (50 000 total V·h). To check the reproducibility, all the protein samples were analyzed by 2-DE in triplicates.

Results

Production of sHsps. The sHsps were produced in recombinant *E. coli* in the polyhistidine-tagged form to facilitate purification. Plasmids pTac99IbpA*his*$_{Ec}$, pTac99IbpB*his*$_{Ec}$, and pTac99Hsp26*his*$_{Sc}$ were used for the production of polyhisti-

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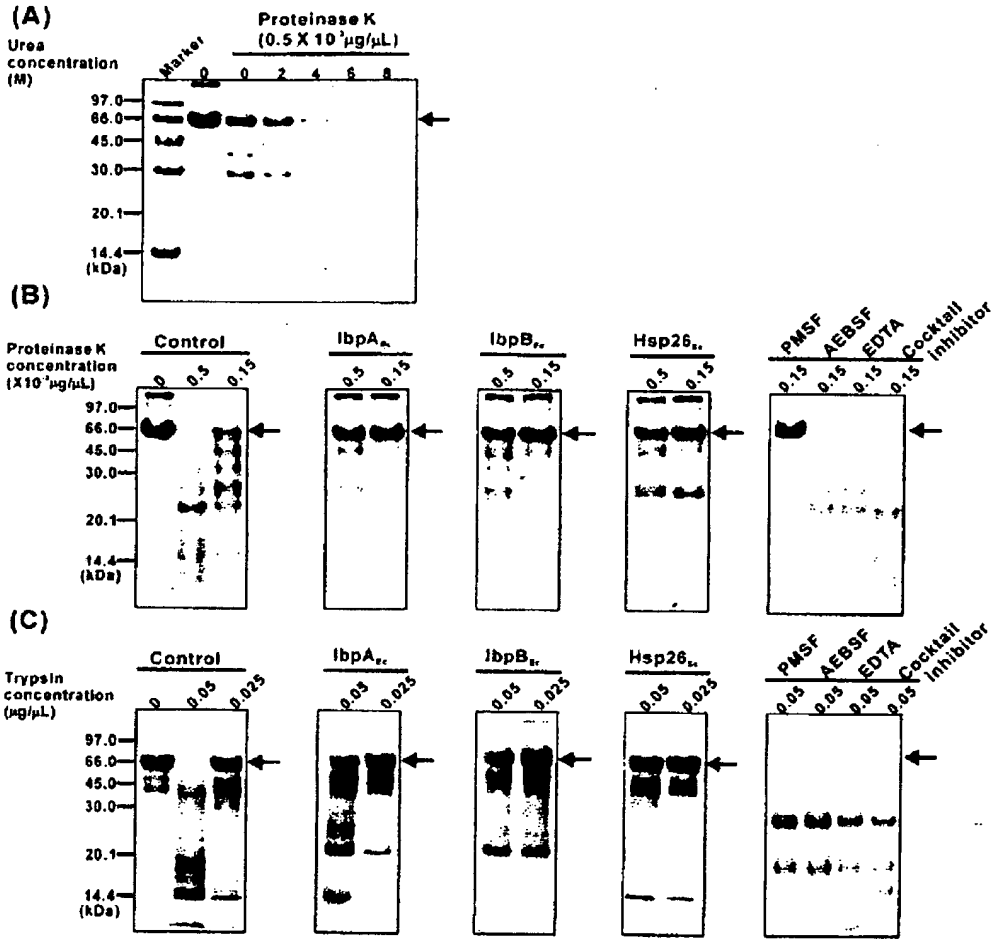

Figure 1. Effects of sHsps on in vitro proteolysis. (A) The susceptibility of HSA to proteolytic cleavage by proteinase K in the presence of 0–8 M urea. HSA (0.5 μg/μL) was incubated with 0.5 × 10^{-3} μg/μL of proteinase K and various concentrations of urea for 2 h at room temperature. (B and C) Inhibition of proteolysis by sHsps. HSA (0.5 μg/μL) was incubated for 2 h at room temperature with various concentrations of proteinase K (B) or trypsin (C) in the presence of 8 M urea and 0.005 μg/μL of $IbpA_{Ec}$, $IbpB_{Ec}$, or $Hsp26_{Sc}$. Also, currently available protease inhibitors, including 1 mM PMSF, 4 mM AEBSF, 1 mM EDTA, and 0.1 tablet/mL of protease inhibitor cocktail were examined for comparison. Shown on the left are the molecular weight standards, which included phosphorylase b (97 kDa), bovine serum albumin (66 kDa), chicken egg white ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa). Results are representative of triplicate experiments. The arrow indicates the position of the HSA band.

dine-tagged $IbpA_{Ec}$, $IbpB_{Ec}$, and $Hsp26_{Sc}$, respectively. These sHsps were successfully produced in recombinant *E. coli* XL1-Blue after induction with IPTG. $IbpA_{Ec}$, $IbpB_{Ec}$, and $Hsp26_{Sc}$ were produced up to 30%, 40%, and 34%, respectively, of the total *E. coli* proteins (see Supporting Information Figure S1A online). We initially attempted to purify sHsps using a Ni-nitrilotriacetic acid spin column under native conditions but were not successful, probably because the polyhistidine residues in the sHsps were buried.[16] The sHsps were therefore purified under denaturing conditions. The purity of the sHsps as determined by SDS-PAGE was greater than 99% (see Supporting Information Figure S1B online).

Inhibition of Proteolysis In Vitro by sHsps. Solutions of urea are commonly used for protein purification, unfolding/refolding, and separation by IEF during 2-DE. Unfortunately, proteins are more susceptible to proteolysis in their nonnative or denatured state than in their native state. Because many proteases are more resistant to denaturation than other cellular proteins, they can remain active under standard denaturing conditions.[1,2] Thus, the denaturing conditions can enhance the proteolytic degradation of cellular proteins by exposing buried residues while proteases remain active.

We first examined the susceptibility of native and denatured HSA (0.5 μg/μL) to proteolysis by proteinase K in the presence of 0 to 8 M urea. As expected, HSA became more susceptible to proteolysis as the urea concentration was increased (Figure 1A). Next, we examined the effects of sHsps on the degradation of HSA by proteinase K in the presence of 8 M urea (Figure

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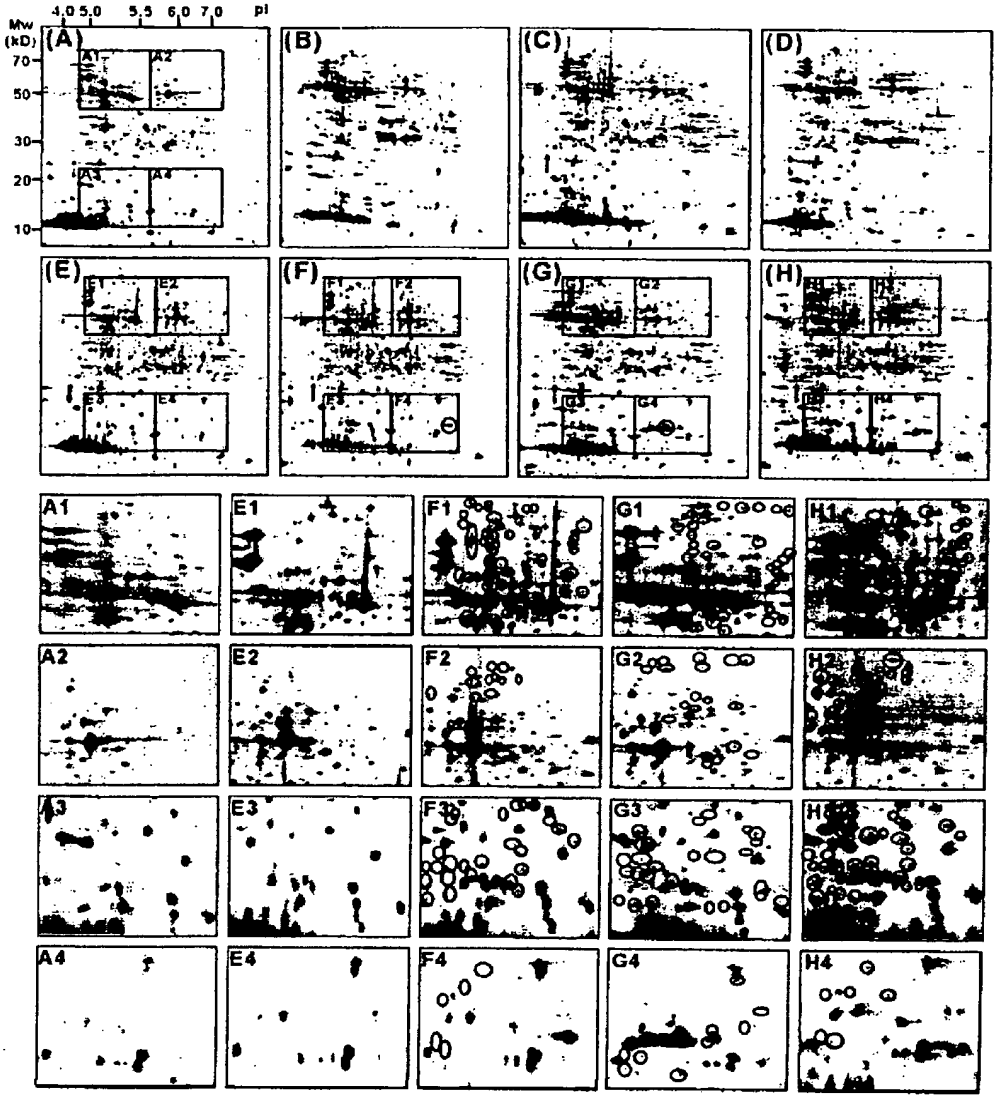

Figure 2. 2-DE profiles of *E. coli* whole cell lysate under various conditions. Gels were loaded with *E. coli* whole cell lysate (200 μg of proteins) treated with various protease inhibitors or sHsps. IEF was carried out using the pH 3–10 NL IPG strips for the first dimension of separation, and SDS-PAGE on a 12% polyacrylamide gel was performed as the second dimension of separation. The gels were silver stained, scanned, and the protein spots were detected by digital imaging software. (A) No inhibitor; (B) 1 mM PMSF; (C) 4 mM AEBSF (Pefabloc SC); (D) 1 mM EDTA; (E) 0.1 tablet/mL of protease inhibitor cocktail; (F) 5% (10 μg) $IbpA_{Ec}$; (G) 5% (10 μg) $IbpB_{Ec}$; (H) 5% (10 μg) $Hsp26_{Sc}$. Circles in F, G, and H indicate the spots of $IbpA_{Ec}$, $IbpB_{Ec}$, and $Hsp26_{Sc}$, respectively. Lower panels show enlarged sections selected for comparison. Circles in the lower panels indicate new protein spots which could not be seen in the controls.

1B). Proteinase K was added at varying concentrations to 0.5 μg/μL. HSA without or with sHsps ($IbpA_{Ec}$, $IbpB_{Ec}$, or $Hsp26_{Sc}$) or commercial PIs. All three sHsps were able to protect HSA from proteolysis by proteinase K. Notably, as little as 0.005 μg/μL of the sHsps effectively inhibited proteolysis by proteinase K. Of the commercial PIs tested, including PMSF, AEBSF, EDTA, and a commercial protease cocktail inhibitor, only PMSF was able to protect HSA from proteolysis. Similar experiments were carried out using trypsin as another model protease (Figure 1C). Again, addition of $IbpA_{Ec}$, $IbpB_{Ec}$, or $Hsp26_{Sc}$ protected HSA from proteolysis. None of the commercial PIs was able to inhibit proteolysis, suggesting that sHsps are much more effective than the commonly used PIs at protecting HSA from proteolysis by trypsin.

Enhanced 2-DE of *E. coli* Cell Extract by the Addition of sHsps. The above findings suggested that sHsps may be able to solve some of the problems caused by proteolysis during 2-DE. We first determined the optimal concentration of sHsps for the inhibition of proteolysis during 2-DE of the whole cell lysate of *E. coli* W3110 as a model proteome. When sHsps were

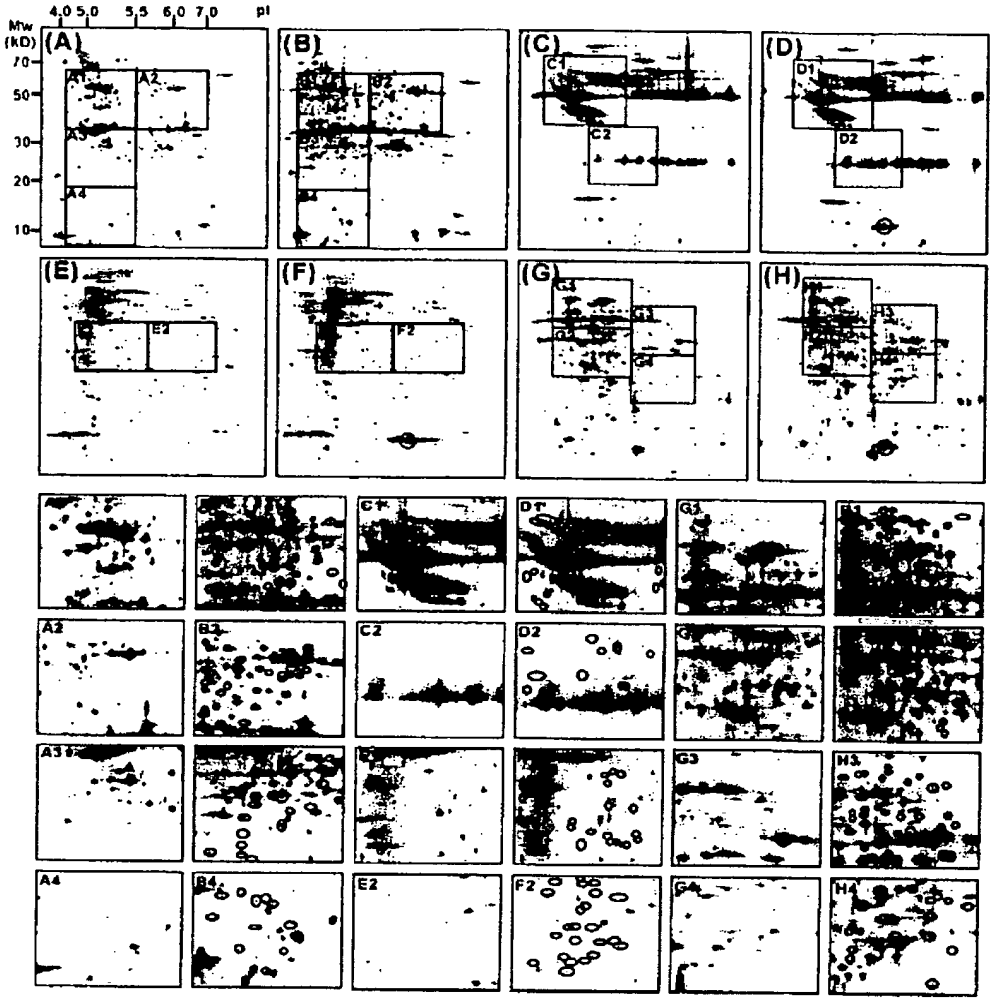

Figure 3. 2-DE profiles of *M. succiniciproducens* cell extract (A,B), human serum (C,D), human kidney cells (E,F), and *A. thaliana* (G,H). Samples (150 μg of proteins) were mixed with 0.1 tablet/mL of protease inhibitor cocktail (A,C,E,G), 5% (7.5 μg) $Hsp26_{Sc}$ (B), or 5% (7.5 μg) $IbpB_{Ec}$ (D,F,H). Circle in B indicates the $Hsp26_{Sc}$ spot, while those in D, F, and H are $IbpB_{Ec}$ spots. Lower panels show enlarged sections selected for comparison. Circles in the lower panels indicate new protein spots which could not be seen in the controls.

added at below 1% (w/w) of the total loading proteins, there was no positive effect. When sHsps were added at 20% (w/w) or higher percentage of the total loading proteins, problems of overlapping protein spots were observed. The optimal concentration of sHsps was found to be 5% (w/w) of the total protein loaded for 2-DE. We further compared the abilities of the three sHsps and the commonly used PIs to inhibit proteolysis during 2-DE of *E. coli* W3110 whole cell lysate. In all cases, the pH gradient was between 3 and 10, and SDS-PAGE was performed on a 12% polyacrylamide gel. The overall profiles of cellular proteins were quite reproducible. As shown in Figure 2, the efficiency of inhibition was as follows: sHsps ≈ protease inhibitor cocktail ≥ AEBSF ≈ EDTA > PMSF ≈ None (see Supporting Information Figure S3 online for higher resolution 2-D gels). When 2-DE was carried out in the absence of PIs or sHsps, we detected approximately 900 protein spots (Figure 2A); in the presence of AEBSF (Figure 2C) or the protease inhibitor cocktail (Figure 2E), this increased to approximately 1200 spots; and when $IbpA_{Ec}$ (Figure 2F), $IbpB_{Ec}$ (Figure 2G), or $Hsp26_{Sc}$ (Figure 2H) was added, we detected 1600, 1700, and 1700 spots, respectively. The lower panel of Figure 2 shows an enlargement of several small regions from representative gels. This reveals that the addition of $IbpA_{Ec}$, $IbpB_{Ec}$, or $Hsp26_{Sc}$ not only increased the number of spots but also enhanced their definition and intensity. Furthermore, addition of the sHsps prevented the loss of both high and low molecular mass proteins. Therefore, our results indicate that the addition of sHsps significantly improves 2-DE-based proteome analysis.

General Improvement of Proteome Profiling by sHsps. To determine whether this strategy can be generally applied, we examined the effects of adding sHsps to extracts from another bacterium (capnophilic bovine rumen bacterium *Mannheimia succiniciproducens* MBEL55E), human serum, and extracts of

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human kidney cells and plant cells (Figure 3 and see Supporting Information Figure S4 online for higher resolution 2-D gels). The overall profiles of cellular proteins taken from each sample within the pH range of 3−10 were quite reproducible. $Hsp26_{Sc}$ or $IbpB_{Ec}$, and protease inhibitor cocktail was compared as a representative sHsp and a strong protease inhibitor currently available, respectively. The number of spots obtained for *M. succiniciproducens* in the presence of $Hsp26_{Sc}$ was approximately 1100, whereas 740 spots were obtained when the protease inhibitor cocktail was used (Figure 3A,B; enlarged images of selected sections are shown in Figure 3A1-4,B1−4). For human serum, approximately 710 spots were obtained in the presence of $IbpB_{Ec}$, whereas 600 spots were obtained when the protease inhibitor cocktail was used (Figure 3C,D). Enlarged images of selected sections clearly show more protein spots when $IbpB_{Ec}$ was added (Figure 3D1-2 compared with Figure 3C1-2). Similar improvements in the number and quality of the protein spots were observed when $IbpB_{Ec}$ was added to the human kidney cell line extract (1462 and 1642 spots in Figure 3E and 3F, respectively; also see Figure 3E1-2,F1−2). Finally, we examined the effect of the sHsps on the proteome profiling of the extract of *Arabidopsis thaliana* Colombia as a model plant sample. This was particularly interesting because plant cell extracts are generally rich in proteases.[16] As shown in Figure 3G and H (1852 and 2265 spots, respectively), the number of protein spots observed was significantly higher when $IbpB_{Ec}$ was added. Taken together, these results indicate that sHsps can universally protect proteins from proteolysis in vitro and that they are exceptionally efficient in enhancing the 2-DE based proteome analysis.

Discussion

Because of proteolytic degradation of proteins in cellular extracts, the proteome profiles obtained by 2-DE have not been truly representing all the proteins in the cell. Even though the addition of various protease inhibitors can improve the performance of 2-DE to some extent, proteolysis still persists during the IEF step.[12] This loss or modification of protein spots can be a substantial problem when the entire protein content of a sample must be analyzed. In this report, we showed for the first time that some of these problems can be solved by simply adding sHsps. The use of sHsps for the inhibition of proteolysis has three major advantages over commercially available PIs. First, they are extremely effective for the inhibition of proteolysis, allowing the detection of protein spots that cannot be detected using currently available PIs. Second, sHsps are much less expensive than currently available PIs: the sHsps can be produced in large amounts by cultivation of recombinant *E. coli* and can be purified easily in 8 M urea without further processing. Third, because the sHsp is added at a known concentration, it can be used as an internal standard for spot quantitation in 2-DE. It is believed that this new approach will change the way 2-DE is practiced because it allows detection of up to 50% more protein spots than those detectable with the best protease inhibitors currently available. Therefore, many more protein spots that could not be seen before can now be detected by the use of sHsps. In addition, sHsps can be employed for protein purification or other bioprocesses where proteolysis is a problem. In conclusion, the novel finding that sHsps can be effective protease inhibitors in vitro should be useful for various applications including proteomics.

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Supporting Information Available: Information on gene manipulation, Figure S1: The expression (A) and purification (B) of polyhistidine-tagged $IbpA_{Ec}$, $IbpB_{Ec}$, and $Hsp26_{Sc}$ from recombinant *E. coli* XL1-Blue harboring pTac99Ibp*A*/*his*$_{Ec}$, pTac99Ibp*B*/*his*$_{Ec}$, and pTac99Hsp26/*his*$_{Sc}$, respectively, Figure S2: Circular dichroism of $IbpB_{Ec}$ after refolding, Figure S3: The enlarged 2-DE profiles of *E. coli* whole cell lysate from Figure 2, and Figure S4: The enlarged 2-DE profiles *M. succiniciproducens* cell extract (A,B), human serum (C,D), human kidney cells (E,F), and *A. thaliana* (G,H) from Figure 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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